SOME FACTORS AFFECTING THE STABILITY OF SKIN TISSUE FAT

by

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INTRODUCTION

The problem of rancidification in fats and oils and its prevention has been the subject of a great number of studies during the past two decades. Until about 1935 most of these investigations were concerned with enhancing the stability of extracted fats and oils. However, since that time the more difficult problem of preventing rancidity in the fat of meat and other whole tissues has received more and more attention due to the advent of cold storage as a means of food preservation.

The work done prior to 1947 established two important factors which affect the stability of fats. These are (a) the fatty acid composition and (b) the amount and nature of existing natural anti or pro-oxidants. Since both of these factors have been shown to be affected by the nature of the dietary fat, most of the work has been directed along this line. For example, the effect of varying degrees of saturation in dietary fats on the fatty acid composition of the skin tissue fat of chickens has been shown clearly by recent work of Kummerow (1). The skin tissue fat from birds which had received a diet consisting of 25 percent hydrogenated fat (Spry) had a fatty acid composition of 34.4 percent saturated, 48.7 percent oleic, and only 16.62 percent linoleic; whereas birds receiving the same basal ration containing 25 percent linseed oil had a fatty acid composition of 13.3 percent saturated, 39.1 percent oleic, 21.35 percent linoleic, and 25.8 percent linolenic. The induction period of the skin fat from the Spry fed group was 168 hours at 70 degrees

compared to 4 hours for the fat from the linseed oil group. Schreiber, Vail, Conrad, and Payne (2) have also shown that the feeding of unsaturated fatty acids in the form of fish oil for as little as two weeks before slaughter decreased the stability of the carcass toward oxidative rancidity during cold storage.

Decreasing the total unsaturation of skin tissue fats by feeding highly saturated fats would seem to be the answer to the problem of oxidative rancidity in cold storage if it were not for the fact that the higher unsaturated fatty acids are essential in the diet for proper growth and metabolism. The Spry fed chickens of Kummerow (1) developed crooked legs and oily feathers and did not gain as much weight as did the control birds which received 25 percent corn in their ration. The essential character of linoleic and arachidonic acid has also been shown by the work of Burr (3). For this reason, most of the work on the stabilization of tissue fats has been directed toward increasing the concentration of naturally occuring antioxidants in the fat depots.

The most promising of the naturally occurring antioxidants found thus far are the tocopherols. Lundberg et al. (4) have found that the concentration of tocopherol in the fat depots of the white rat could be increased by increasing the amounts of tocopherol fed. The induction period of the body fat was found to be a parabolic function of the amount of tocopherol fed, which indicates that there is an ultimate limit to the amount of tocopherol which can be stored in the fat depots. This group of investigators (5) also has made some studies of butterfat

stability as related to diet. The effect of tocopherol was tested by adding 6.2 g of synthetic alpha tocopherol each week to the vitamin E free basal diet of a cow. The control received only the basal diet. Butterfat from these two groups of animals was tested in Warburg microrespirometers at 100 degrees. The induction period of the E-free butterfat was essentially zero, while that from the E-fed cow was distinctly increased, an additional 30 minutes being required before full rate of oxygen uptake began. In work with hogs, Watts et al. (6) fed a distillation mixture consisting of 34 percent mixed tocopherols and found only small decrease in the susceptibility of their fat to oxidative rancidity. However, the amounts of tocopherol fed in relation to the weight of the animals did not approach the larger dosages fed by Lundberg et al. (4).

The group at the University of Minnesota has also pointed out some interesting facts concerning the relative effectiveness of the individual tocopherols in stabilizing fats. The alpha and beta forms were found to be about twice as effective as the gamma in stabilizing body fat when fed to rats, but when added directly to rendered body fat the gamma was several times as effective as an antioxidant. Hove and Hove (7) have confirmed the finding that gamma tocopherol is the superior antioxidant at the induction period temperature (100 degrees) used by Lundberg et al., but have also found that at low temperatures (25 to 35 degrees) the three tocopherols exhibit approximately equal antioxidant activity. In the light of this evidence it would appear that the body can utilize alpha tocopherol and store it in the

fat depots as the gamma form, but cannot utilize the gamma form and store it in the fat as such. The metabolism of the tocopherols will be better understood when better methods of quantitatively determining the individual tocopherols in fats are developed.

Another group of compounds which occur naturally in animal fats are the phospholipids. Normally they make up from 2.5 to 3.2 percent of the total lipid in skin tissue. Conflicting evidence can be found in the literature concerning their effectiveness as antioxidants. Lea (8) states that some workers have obtained antioxidant protection factors of 1.5 to 3.0 and 1.7 to 1.8 when concentrations of 0.1 to 0.2 percent were used; while other observers failed to obtain any effect by the addition of relative large amounts of phospholipid. Such conflicting evidence can be better understood when more recent observations on the antioxidant activity of the phospholipids are considered. Olcott and Mattill (9) have showed that various commercial preparations were more effective as antioxidants in vegetable oils than in animal fats or purified fatty acids or esters, although marked activity could be demonstrated in the latter substrates, if tocopherols or other phenolic inhibitors were added. Purified lecithin was inactive, but the activity appeared quantitatively in the cephalin fraction.

The objective of the present investigation was to study the variations in vitro in the stability of skin tissue fat caused by varying the concentration of various substances which are naturally deposited in this fat. This work is especially important

in determining the degree of stability which might be expected when poultry rations are supplemented with varying amounts of these natural antioxidants in vivo.

EXPERIMENTAL

Preparation of Materials

The skin tissue and subcutaneous fat of a mature (16 months) chicken of the Plymouth Rock breed, raised under normal conditions on a standard commercial ration, was extracted by the method of Bloor (10, p. 40) as modified in this laboratory.

For the experiments with tocopherol an attempt was made to prepare the methyl esters of the unsaturated fatty acids occurring in chicken fat in a pure form. Pure methyl oleate was available in the laboratory. Also at hand were the polybromides of linoleic and linolenic acids from which the pure methyl esters of the acids were prepared. An impure sample of ethyl arachidonate was also available and was purified via the octobromide route. The saturated fat used in preparing the synthetic chicken fat was prepared by exhaustive hydrogenation of the saturated fatty acids of olive oil to an iodine value of zero. For the other phases of the work the esters were prepared by cold interesterification of chicken fat with methanol using the method of Kurz, according to Markley (11, p. 582). Samples of pure alpha and beta tocopherol were obtained from the Distillation Products Company, Rochester.

The technique used for the preparation of these compounds and the methods of making various determinations are given in

some detail in the following pages.

Extraction of Skin Tissue Fat. The weighed tissue was cut into small pieces, placed in Erlenmeyer flasks with enough acetone to cover, and refluxed for one hour. The acetone was then filtered off through a Buchner funnel and the extraction with acetone repeated. The filtered acetone extractions were combined and the tissue extracted again in the same manner using 95 percent alcohol. The alcohol extractions were combined and filtered through anhydrous sodium sulfate into a weighed round-bottom flask. By the same procedure the tissue was extracted twice with Skellysolve F and the acetone and Skelly extracts combined in a separatory funnel and shaken. The Skellysolve layer was drawn off and the acetone layer washed several times with fresh Skellysolve. The combined Skellysolve extracts were washed three times with equivalent volumes of water and dried by filtering through anhydrous sodium sulfate. The solvent was removed from the alcohol extractions under vacuum and the residue taken up with the dried Skellysolve extract. The Skellysolve was then removed under vacuum until only the lipid and lipid soluble substances present in skin tissue fat remained. The flask was weighed and the amount of fat extracted was determined.

Preparation of Methyl Linoleate. Two hundred g of tetrabromostearic acid obtained by bromination of corn oil and 200 g of granular zinc were mixed together and placed in a dry, 1 liter, round bottom flask. A condenser was attached and 200 ml of methyl alcohol were added and the acid dissolved by warming care-

fully on a steam bath. Three hundred ml of a 3 normal solution of dry hydrochloric acid in methyl alcohol were cautiously added, and the flask carefully rotated to start the reaction. Cooling in a stream of tap water was necessary to control the initial reaction, after which the mixture was shaken vigorously and allowed to reflux for 2 hours. The mixture was then cooled and poured into a separatory funnel containing 200 ml of water and 200 ml of Skellysolve B. The mixture was shaken and the aqueous layer drawn off and shaken again with 200 ml of Skellysolve B. The aqueous layer was then discarded. The Skellysolve extracts were combined and washed with an equal volume of water, care being taken to avoid emulsification. After drying the Skellysolve extract by filtering through anhydrous sodium sulfate, the Skellysolve was removed under vacuum. The methyl linoleate was then distilled in a vacuum still and sealed under vacuum in test tubes until ready for use. Its iodine value was 165.9; theoretical being 172.4. Its peroxide value was zero.

Preparation of Methyl Linolenate. Two hundred g of hexabromostearic acid were treated in the same manner as the tetrabromostearic used in preparing methyl linoleate except for the fact that 24 hours of refluxing were required for complete debromination instead of 2 hours. The methyl linolenate was then sealed under vacuum in test tubes until ready for use. Its iodine value was 244.4; theoretical being 260.4. Its peroxide value was zero.

Bromination of Impure Ethyl Arachidonate. A 5 percent solution of the impure ester in ether was placed in a 500 ml flask

and clamped firmly into an ice-salt bath. The flask was at least three inches above the bottom of the bath to provide proper cooling. An air jet stirrer with sufficient speed and power to produce good mixing was adjusted to about one-half inch from the bottom of the flask. Bromine was added from a separatory funnel at such a rate that the temperature of the reaction mixture at no time exceeded 10 degrees. When a faint orange color of free bromine persisted in the reaction mixture, the addition of bromine was stopped, the flask corked tightly, and allowed to stand overnight at -5 degrees. The crude octabromide was then collected on a Buchner funnel in the cold room, and washed repeatedly with redistilled ether until it had a snow-white appearance. The purified ethyl octobromostearate was then sucked dry on the filter, and removed to a vacuum desiccator to completely dry it.

Preparation of Ethyl Arachidonate. Fifteen g of ethyl octabromostearate and 15 g of powdered zinc were mixed together and placed in a dry, 250 ml, ground-neck, round-bottom flask. A condenser was attached, and 100 ml of a 3 normal solution of dry hydrochloric acid in absolute alcohol were added cautiously. The mixture was refluxed for 8 hours, then cooled, and poured into a separatory funnel containing 50 ml of water and 50 ml of Skellysolve F. After shaking, the aqueous layer was drawn off and extracted again with another 50 ml of Skellysolve. The aqueous layer was then discarded, and the Skellysolve extracts combined and washed with an equal volume of water, care being taken to avoid emulsification. After drying the Skellysolve extract by filtering through anhydrous sodium sulfate, the Skellysolve was

removed under vacuum. The ethyl arachidonate was then distilled in a molecular still, and sealed under vacuum in test tubes until ready for use. Its iodine value was 303.5; theoretical being 305.3.

Preparation of Saturated Methyl Esters of C_{16} and C_{18} Acids. A 10 percent solution of olive oil in acetone was allowed to stand overnight at -15 degrees. Twenty g of the fat which crystallized at this temperature was collected on a Buchner funnel, and transferred to a 500 ml flask which contained 200 ml of a 10 percent solution of potassium hydroxide in 95 percent alcohol. A condenser was attached, and the solution refluxed for 1 hour. The saponification mixture was then cooled and poured into a separatory funnel containing 100 ml of water and 100 ml of Skellysolve F. The mixture was shaken, and the aqueous layer separated and discarded. The Skellysolve layer was dried by filtering through anhydrous sodium sulfate, and the solvent removed under vacuum. The methyl esters were dissolved in sufficient amount of a mixture (2:1) of 95 percent alcohol and Skellysolve F to make a nearly saturated solution. This solution was then poured into a hydrogenation bottle which contained 5 g of freshly prepared Raney nickel catalyst, and the bottle clamped firmly into a mechanical shaker. Dry hydrogen gas under 30 pounds of pressure was admitted into the bottle through the cork, and the mixture shaken under this pressure for 72 hours. The catalyst was then filtered off, and the solvent removed under vacuum. The resulting methyl esters had an iodine value of zero.

Cold Interesterification of Chicken Fat with Methanol. Fif-

ty parts by volume of a 20 percent solution of chicken fat were mixed with 35 parts of neutralized methanol and 1.0 part of 0.5 N solution of potassium hydroxide in methanol and allowed to stand for 24 hours at room temperature. Sufficient water was then added to separate the methanol from the ether, and the mixture poured into a separatory funnel. The aqueous phase was drawn off and discarded. The ether phase was dried with anhydrous sodium sulfate, and the ether removed under vacuum. The methyl esters which remained still contained the glycerol and the natural pigments which occur in chicken fat. Vacuum distillation of the esters showed that the interesterification was more than 98 percent complete. The fatty acid composition of the esters was essentially the same as that of the chicken fat.

Preparation of Trilinolein, Method of Black and Overley (12). Eighty g of tetrabromostearic acid in a round-bottomed flask under a reflux condenser was placed in an oil-bath at 120 degrees. When the acid had melted, 15 g (1.2 mole) of thionyl chloride was added in small portions through the condenser over a period of one hour. Only slight darkening occurred. The mixture was allowed to cool to room temperature and stand overnight. The oil was then taken up in 250 ml of anhydrous petroleum ether and placed at -26 degrees for several hours. The shiny, white plates which separated were filtered in the freezer and washed with cold petroleum ether until most of the excess thionyl chloride was removed. The product was carefully dried in vacuo. Its melting point was 59 to 60 degrees; theoretical for 9, 10, 12, 13-tetrabromostearoyl chloride being 59.5 to 60.0 degrees.

To a cold mixture of 2.2 g (0.024 mole) of glycerol and 9.6 g (0.075 mole) of quinoline in a glass-stoppered Erlenmeyer flask was added, with shaking and cooling, 45 g (0.073 mole) of 9, 10, 12, 13-tetrabromostearoyl chloride in 30 ml of dry chloroform. The semi-solid mass was allowed to stand at room temperature for two days, and taken up in 600 ml of ether, and washed successively with 0.5 N sulfuric acid, saturated sodium bicarbonate solution, and water. The ether solution was dried over anhydrous sodium sulfate and placed at 0 degrees. The crystals which separated were recrystallized from ether.

The debromination of the tri-(9, 10, 12, 13-tetrabromo)-stearin was carried out by treating 20 g lots of the tetrabromo derivative with 20 g of 30-mesh zinc and 50 ml of absolute alcohol. The zinc had been washed with dilute hydrochloric acid, then with water, and carefully dried. The flask was warmed gently in a water-bath until the beginning of the vigorous exothermic reaction, which was controlled by cooling the flask with ice. Finally the alcohol was refluxed for half an hour, and the zinc removed by filtration. The flask and zinc were washed with 150 ml of anhydrous petroleum ether. The filtrate and washing were washed several times with water, and finally dried over anhydrous sodium sulfate and evaporated in vacuo. The nearly colorless oil was taken up in 30 ml of a 1:1 ether-petroleum ether mixture and let stand at -26 degrees overnight. The product did not crystallize at this temperature. After molecular distillation the fraction which did not distill had the following constants: iodine value, 166; calculated for trilinolein, 173; saponification value, 185; calculated, 191.

Separation of the Phospholipids from Chicken Fat. Two methods were used. In the first method 50 g of chicken fat were placed in a 250 ml centrifuge bottle, and a mechanical stirrer adjusted ½ inch from the bottom of the bottle. The bottle was then filled with acetone, and stirred at room temperature for 1 hour. The phospholipids which precipitated were separated from the fat by centrifuging at 1800 rpm for 15 minutes. The acetone extract was decanted, and the phospholipids immediately dissolved in 50 ml of Skellysolve F to prevent their autoxidation.

In the second method 10 g of chicken fat were placed in a 250 ml centrifuge bottle containing 5 cc of distilled water. A mechanical stirrer was inserted, and the mixture stirred at room temperature for ½ hour. A fine emulsion resulted. The emulsion was broken by centrifuging at 1800 rpm for 15 minutes. The bottle containing the separated fat and water phases was allowed to stand at -26 degrees for 1 hour, and the fat scraped off of the ice layer with a spatula. The ice phase was then chipped out of the bottle with a porcelain spatula and placed in a vacuum desiccator under a pressure of 20 microns for 2 days. When the vacuum was broken, the dried phospholipids were immediately redissolved in chicken fat and the induction period of the fat determined.

Determination of Constants

Determination of Fatty Acid Composition of Chicken Fat. A modification of the spectrophotometric method of Kraybill, Mitchell, and Zscheile (13) was used. Duplicate samples of fat, ap-

proximately 0.1 g in weight, were weighed accurately in small glass weighing vessels. These vessels were then dropped into 25 x 275 mm pyrex test tubes containing 5 ml of 7.5 percent potassium hydroxide in ethylene glycol which previously had been brought to a constant temperature of 180 degrees by immersion in a constant temperature oil bath. The fat samples were isomerized at this temperature for 30 minutes under a constant stream of nitrogen. At the end of this period the test tubes were removed and placed in a beaker of cold water to stop the reaction. The contents of the tubes were then quantitatively transferred to 100 ml volumetric flasks, and made up to volume with redistilled 95 percent ethanol. The diluted samples were kept overnight at 10 degrees to precipitate any silica removed from the test tubes by the alkaline reagent. The next morning the samples were allowed to come to room temperature and filtered. Appropriate dilutions were then made to bring the optical density of the solutions to values between 0.500 and 0.800. The optical densities of the solutions were recorded at wave lengths of 2320, 2340, 2620, 2680, 2740, 3100, 3160, and 3220 Angstroms. Dilutions were recorded along with the optical densities. Two blanks were run along with each isomerization.

Appropriate dilutions also were made on unisomerized samples, and optical densities were recorded at the above wave lengths. Fatty acid percents were calculated as follows:

$$K_2 = \frac{R_{2320}}{g/liter} - 0.07$$

$$K_3 = \frac{2.8}{g/liter}\left[R_{2680} - \tfrac{1}{2}(R_{2620} + R_{2740})\right]$$

$$K_4 = \frac{2.5}{g/liter}\left[R_{3100} - \tfrac{1}{2}(R_{3100} + R_{3220})\right]$$

Unisomerized samples (used as correction factors)

$$K'_2 = \frac{R'_{2320}}{g/liter} + .04$$

$$K'_3 = \frac{4.1}{g/liter}\left[R'_{2680} - \tfrac{1}{2}(R'_{2620} + R'_{2740})\right]$$

$$K'_4 = \frac{2.5}{g/liter}\left[R'_{3160} - \tfrac{1}{2}(R'_{3100} + R'_{3220})\right]$$

Isomerized samples

$$K''_2 = K'_2 - K_2$$

$$K''_3 = K'_3 - K_3$$

$$K''_4 = K'_4 - K_4$$

Percent linoleic acid = $1.125 \times K''_2 - (1.27 \times K''_3)\ 0.04 \times K''_4$

Percent linolenic acid = $1.87 \times K''_3 - 4.43 \times K''_4$)

Percent arachidonic acid = $4.43 \times K''_4$

Percent oleic acid = iodine value x 100 - (181.5 x %linoleic acid) + (273.5 x % linolenic acid) + (333.5 x % arachidonic acid / 90)

Percent saturated acid 100 - sum of the above percents

Determination of Iodine Value. Approximately 0.1 g of fat was accurately weighed into a glass-stoppered 125 ml flask, followed by the addition of 5 ml of chloroform. When the sample was

dissolved, 15 ml of Wijs solution was added with a volumetric pipette. The flask was stoppered tightly and placed in the dark for exactly 1 hour. Ten ml of 15 percent potassium iodide was added and mixed well. The stopper and sides of the flask were washed down with 5 ml of distilled water. The solution was titrated with 0.1 N sodium thiosulfate to a faint yellow, a few drops of starch indicator solution were added, and the solution titrated to a clear white. Two blank samples containing no fat were titrated along with each group of unknowns.

The Wijs solution was made as follows: To one liter of acetic acid 13 g of iodine were added and heated until dissolved. After cooling, chlorine gas was bubbled through the solution until a light orange-red color developed. Sufficient chlorine should be added to double the titration of the original iodine solution, but little chlorine in excess of this requirement can be present.

To standardize the thiosulfate solution, the following procedure was used: Ten ml of exactly 0.1 N potassium dichromate and 5 ml of concentrated hydrochloric acid were placed in a 125 ml flask. Ten ml of 15 percent potassium iodide was added, and the mixture titrated to a green color with thiosulfate solution. Three drops of starch indicator were added, and the titration continued to a clear solution.

The calculations were as follows:

$$\frac{126.9 \text{ (.1 for normality of } K_2Cr_2O_7)}{\text{(ml of thio used in standardization)}} = \text{Normality factor}$$

$$\frac{(\text{Normality factor}) \quad (\text{Blank titration minus sample titration})}{\text{Sample weight in g}} = \text{Iodine number}$$

Determination of Peroxide Number. Approximately 0.1 g of fat was accurately weighed into a 125 ml glass-stoppered flask and dissolved in 10 ml of a 1:1 mixture of chloroform and acetic acid. One ml of a saturated potassium iodide solution was added from a pipette. The flask was stoppered securely and shaken for exactly one minute. The stopper and sides of the flask were washed down with 5 ml of distilled water. A few drops of starch indicator were added, and the mixture immediately titrated with .01 N thiosulfate solution until chalky white. The peroxide number was calculated as follows:

$$\frac{0.1 \text{ (for normality of } K_2Cr_2O_7) \times 2}{\text{ml of thio. used in standardization}} = \text{Normality of thiosulfate}$$

$$\frac{(\text{ml of titration}) \times (\text{Nor. of thio.}) \ (0.5) \ (1000)}{\text{Sample weight in g}} = \text{Peroxide number}$$

Determination of Induction Periods. The induction periods were determined with the apparatus pictured in Plate I. One-gram samples of fat were weighed in duplicate into ten 125 ml iodine flasks with funnel type necks. Each flask was then stoppered with a small mercury manometer constructed on a male joint which fit the ground glass neck of the flask. The manometer consisted of three arms, one of which opened into the flask, the other two opening onto electrical contacts which completed a circuit through a recorder as long as the mercury in all three arms of the manometer was at the same level. After the manometers were filled with mercury, the flasks were suspended from a rack into a con-

EXPLANATION OF PLATE I

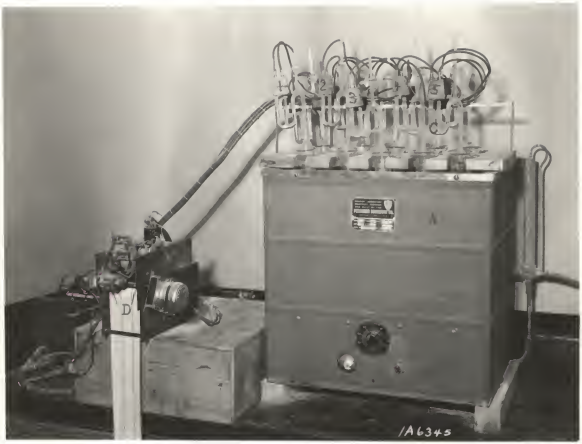
A. Constant temperature water bath.

B. Funnel neck, 125 ml iodine flask.

C. Mercury manometer.

D. Automatic recorder.

PLATE I



stant temperature water bath, and the electrical contacts inserted in the manometer arms. When a temperature equilibrium between the flasks and the bath was reached, the system was closed off by means of stopcocks opening into it. The contacts were adjusted so that the circuit would be broken when enough oxygen was absorbed by the fat in the flask to lower the pressure in the system an amount equivalent to 1 cm of mercury. By means of a clock which made contact with each manometer circuit once every hour, thereby actuating an electronic relay, the time to the nearest hour that each manometer circuit was broken could be measured. The time required to break the manometer circuit was considered the length of the induction period.

RESULTS

The Effect of Increasing the Concentration of Alpha and Gamma Tocopherol on the Induction Period of Synthetic Chicken Fat

A mixture of pure methyl esters having the same fatty acid composition as chicken skin tissue fat was used in these experiments. This substrate was used largely for two reasons. First, gamma tocopherol was found to impart a very high degree of stability to the natural chicken fat, the time required for oxygen uptake to begin being so long that it was considered prohibitive under the conditions of the present experiments; and second, the use of a pure substrate permits the absolute effect of the tocopherols to be determined.

The results obtained using four different concentrations of

alpha and gamma tocopherol are shown graphically in Fig. 1. Both tocopherols appear to give maximum stabilities at concentrations near 1 percent. At this concentration the gamma form protected the substrate from being autoxidized about 5 times as long as the alpha form. It was of interest to observe that there were never sharp induction periods in the case of the gamma supplemented fats, as there were for the fats supplemented with alpha tocopherol. When the supplement was the gamma form of tocopherol, the oxygen uptake was very slow, and several days were required for the manometer mercury to move far enough to break the electrical circuit which actuated the recorder.

This fact also was observed in some preliminary work in which the natural chicken fat was used as a substrate. In the data obtained, the gamma form of tocopherol appeared to be even more effective as an antioxidant for the natural fat relative to the alpha form than what is shown by the curves obtained using the synthetic fat as a substrate. The unsupplemented chicken fat had an induction period of 25 hours at 70 C. When supplemented with 0.25 percent alpha tocopherol, it had an induction period of 118 hours; but when the supplement was the same concentration of gamma tocopherol, there was no evidence of oxygen uptake after 288 hours, at which time the experiment was discontinued. When 0.88 percent gamma tocopherol was used, there was only a very slight movement of the mercury in the manometer observed after 432 hours. In both of the latter two cases in which gamma tocopherol was used, the peroxide value of the fat was 50 when the experiments were terminated.

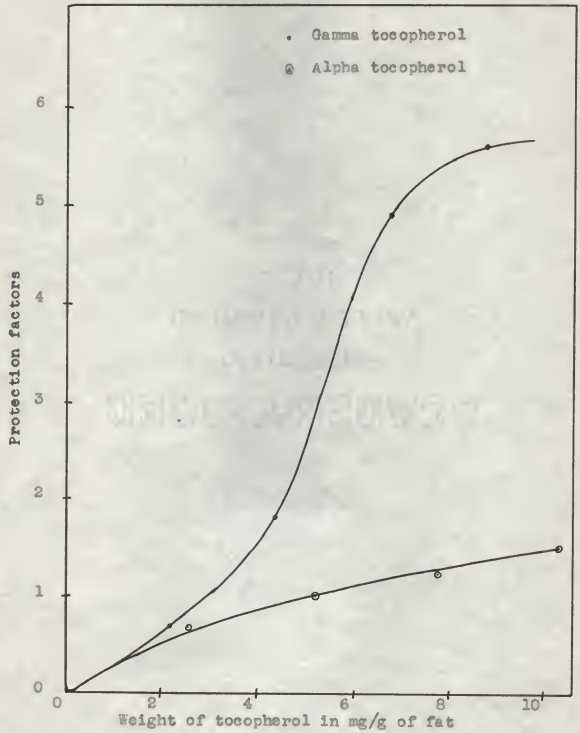


Fig. 1. Protection factor curves for varying concentrations of alpha and gamma tocopherol. Protection factor equals induction period of supplemented fat/induction period of unsupplemented fat. The substrate was a mixture of pure methyl esters having the same fatty acid composition as chicken fat. Temperature, 70° C.

The fatty acid composition of the chicken skin tissue fat and, consequently, of the synthetic chicken fat is given in Table 1. Physical constants for the methyl esters used in preparing the synthetic fat are given in Table 2.

Table 1. The fatty acid composition of the chicken skin tissue fat used in these experiments.

Fatty acid	Percent
Linoleic acid	20.23
Linolenic acid	0.50
Arachidonic acid	0.23
Oleic acid	49.65
Saturated acid	29.39

Table 2. Physical constants of the esters used in preparing the synthetic chicken skin tissue fat.

Ester	Observed Iodine No.	Theoretical Iodine No.	Peroxide Value
Methyl linoleate	165.9	172.41	0.0
Methyl linolenate	244.0	260.40	0.0
Ethyl arachidonate	303.3	305.36	0.0
Methyl oleate	86.0	85.62	0.0
Saturated methyl esters	00.0	00.0	0.0

The Effect of Interesterification with Methanol on the Stability of Chicken Skin Tissue Fat

During the work with tocopherol, it was observed that the natural fat was more than 6 times as stable as the synthetic fat, despite the fact that both fats had the same amount and kind of unsaturation. The data contained in the following sections are the results of attempts to determine whether or not this difference in stability is due, totally or partially, to the ester form of the fat or to the natural antioxidants present in the chicken fat.

Table 3. Stabilities of chicken fat and corn oil compared with the distilled and undistilled methyl esters derived from them.

Fat substrates-Gp.1*	Stability factor	Fat substrates-Gp.2*	Stability factor
Chicken fat	1.00	Corn oil	1.0
Esters (distilled)	0.22	Esters (distilled)	0.11
Esters (undistilled)	0.22	Esters (undistilled)	0.20
Corn oil	2.90		

*In each group the stability factors are based on the original fat as having a stability of 1.00.

The data presented in Table 3 show that chicken fat is rendered about 6 times less stable by interesterification with methanol; and that separation by distillation of the esters from the glycerol, natural pigments, and other non-distillable products of the interesterification reaction made no change in the stabil-

ity of the esters. The methyl esters of corn oil were also found to be much less stable than the original oil, but the undistilled esters were about twice as stable as the distilled esters.

Relative Stabilities of Linoleic Acid, Methyl Linoleate, and Trilinolein

The fact that the undistilled methyl esters of chicken fat were no more stable than the distilled esters immediately brings to mind two possible explanations. First, either methyl esters of unsaturated fatty acids are much more unstable toward autoxidation than unsaturated triglycerides or, second, the natural antioxidants that may be present in chicken fat have their activity destroyed during the interesterification reaction.

The present phase of the work was an attempt to confirm or set aside the first explanation. The induction periods of purified linoleic acid, methyl linoleate, and trilinolein were determined at 50 C. These induction periods are given in Table 4.

It is evident from these results that the triglyceride of linoleic acid is no more stable toward autoxidation than is its methyl ester. Whether or not this is true for mixed triglycerides and mixtures of methyl esters of like fatty acid composition is yet to be determined.

Table 4. The induction periods of linoleic acid homologs measured at 50 C.

Substrate (1 g)	Induction period (hours)
Linoleic acid	6
Methyl linoleate	8
Trilinolein	7
Methyl linoleate plus 2.2 mg gamma tocopherol	Over 200

The Effect of the Phospholipids in Skin Tissue Fat on Its Stability

The experimental results given in the preceding section substantiate the second explanation given there for the difference in stability between natural fats and their mono-esters. With this evidence at hand, it was considered desirable to determine what effect the phospholipids of skin tissue fat had on its stability, since what antioxidant activity these compounds have would be destroyed most likely during the interesterification reaction. The results of these experiments are given in Table 5.

The data given in Table 5 show that chicken fat loses over 3 tenths of its original stability when its phospholipids are removed. Also, once the phospholipids are removed, it requires over 10 times the amount of phospholipid originally present in the fat to return it to its original stability. On the other hand, the esters had their stability more than doubled by the addition of the original amount of phospholipid present. This sta-

bility, however, was still only half that of the original fat.

Table 5. The effect on the stability of chicken skin tissue fat of various concentrations of its natural phospholipid.

Substrate (1 gram samples)	Stability factor
Chicken fat	1.00
Chicken fat, phospholipids removed	0.66
Chicken fat, phospholipids removed, plus phospholipids from 1 g of chicken fat	0.69
*Chicken fat, phospholipids removed, plus phospholipids from 5 g of chicken fat	0.62
Chicken fat, phospholipids removed, plus phospholipids from 20 g of chicken fat	1.40
Methyl esters of chicken fat (undistilled)	0.21
Methyl esters of chicken fat (undistilled) plus phospholipids from 1 g of chicken fat	0.49

*The phospholipids in this determination were separated from the chicken fat by emulsification with water. Phospholipid used in other determinations was separated by precipitation with acetone.

DISCUSSION

The Antioxygenic Effects of Alpha and Gamma Tocopherol

The results concerning the relative antioxidant effects of alpha and gamma tocopherol at 70 C. are in agreement with the findings of Hanson et al. (14) and Hove and Hove (7). Hanson and co-workers found that gamma tocopherol is about 3 times as active an antioxidant as alpha tocopherol when added to lard in vitro at 100 C. Hove and Hove found gamma tocopherol to be 1.76 times more effective than alpha tocopherol in protecting aerated ethyl

oleate from autoxidation at 60 C. and 5.1 times more effective at 95 C. In the experiments described here it is shown that the relative effectiveness of alpha and gamma tocopherol depends upon their respective concentrations. At the optimum concentration for both tocopherols, the gamma form was about 5 times as effective as the alpha form in protecting synthetic chicken fat from autoxidation. In the experiments of Hanson et al. and Hove and Hove, the concentrations of the tocopherols used were of the order of 0.20 percent, and no attempt was made to determine the antioxidant effect relative to tocopherol concentration.

The concentration of alpha tocopherol, which gives maximum protection from autoxidation as reported by Lundberg et al. (4) and Golumbic (15), is 5 to 10 times lower than that found in the work reported here. This seeming lack of agreement is due most likely to the differences in fat substrates used. Golumbic has stated that the concentration of tocopherol and the nature and origin of the substrate fat markedly influence the course of the autoxidation reaction. In work with cottonseed oil and lard, Golumbic found that above 0.10 percent concentration the further addition of mixed tocopherols shortened the induction period. Lundberg and co-workers, on the other hand, used the extracted body fats of vitamin E-deficient rats, and found that synthetic alpha tocopherol gave maximum stability to this fat at concentrations near 0.20 percent.

Why greater amounts of tocopherol can be added to synthetic chicken fat without decreasing the efficiency of the antioxidant is a matter of conjecture at the present stage of this work. This

fact does indicate, however, that the optimum concentration of tocopherol for maximum stability is very likely determined by a combination of factors, among which are the presence of other substances which occur naturally in fats, the ester form of the fat, the total unsaturation present in the fat, and the temperature at which the induction periods are measured. The effect of total unsaturation and temperature is demonstrated by the fact that 0.22 percent gamma tocopherol gives methyl linoleate at 50 C. 10 times the protection from autoxidation that it gives synthetic chicken fat at 70 C. It is regretted that insufficient trilinolein was prepared to permit the determination of the relative effect of tocopherol on the pure triglyceride and pure monoester.

No previous work has been done to determine the optimum concentration of gamma tocopherol for maximum stability. However, judging from the present results, this concentration apparently parallels quite closely the optimum concentration for alpha tocopherol.

The absence of sharp induction periods in the case of gamma-supplemented fats brings out an interesting point in reference to Golumbic's work on the autoxidative behavior of animal and vegetable fats. Golumbic found that chroman-5, 6 quinones appear during the autoxidation of vegetable fats but never during the autoxidation of animal fats, even when synthetic alpha tocopherol was added. These antioxygenic o-quinones retarded the accumulation of fat peroxides in vegetable oil after the disappearance of the tocopherols, and the absence of sharp induction periods

in vegetable oil was accredited to the successive action of tocopherol and the chroman-5, 6 quinones. It is now known (16) that vegetable oils contain large amounts of gamma tocopherol (of the tocopherols found in corn oil, 85 percent is the gamma form), whereas there is only a trace of alpha tocopherol in most animal fats. These findings, along with the results of the work reported here, indicate that chroman-5, 6 quinones are the immediate oxidation products of gamma tocopherol but not of the other forms of tocopherol. The tocoquinones, which are the oxidation products of alpha tocopherol, apparently do not possess antioxidant activity in view of the sharp induction periods obtained using this form of tocopherol as an antioxidant.

Effect of Ester Form on the Stability of Fats

The six-fold drop in stability brought about by cold interesterification of chicken fat with methanol can be attributed to one or a combination of two causes. Either the methyl esters formed are much less stable toward oxidative rancidity than are the original triglycerides, or the activity of the natural antioxidants present in chicken fat is destroyed during the interesterification reaction. The results of the work reported here and what little experimental evidence can be found in the literature tend to rule out the first of these causes. Holman and Elmer (17) have found that the maximum rate of oxidation for trilinolein is greater than that for ethyl linoleate by 0.36 mole of oxygen per acid equivalent per 100 hours. This evidence, along with the present findings that trilinolein has a shorter induction

period than methyl linoleate, shows the monoester to be more resistant to autoxidation than the simple triglyceride.

Whether or not the distribution of the various saturated and unsaturated fatty acids in a triglyceride has an effect on the stability of a fat cannot be determined until better methods are found for preparing mixed triglycerides of known composition.

The Effect of the Phospholipid Fraction on Fat Stability

The data in Table 5 show that the phospholipid fraction of natural fats plays a definite role in protecting fat from oxidative rancidity. Chicken fat as extracted was shown to be over 1.5 times more stable than it was after its phospholipids were removed by acetone precipitation. This finding in itself is not surprising, since the phospholipids have been found at different times by various workers to possess antioxygenic properties. However, the results found in the literature of studies made concerning the antioxygenic properties of the phospholipid fraction of fats are in very poor agreement. Feigenbaum (18) has found unrefined lecithin to be effective in the preservation of vitamin A in margarine. Refined lecithin had no antioxidant power. Sollman (19) found lecithin to be more effective than hydroquinone in protecting cottonseed oil from autoxidation in the presence of cobalt oleate. On the other hand, Nakamura and Tomita (20) found the phospholipids of soybean oil to have only weak antioxidant action on soybean oil. In a review of the literature, Bibby (21) states that the phospholipids have little antioxidant activity when appearing alone, but have synergistic action with tocopherols.

The results of the work reported here give a clue to the cause for these variations in the antioxygenic potency of phospholipids. The fact that the stability of the acetone treated fat is not quantitatively restored by adding back the precipitated phospholipids indicates that a change occurs in the chemical makeup of phospholipid or the acetone soluble lipid as a result of their separation. This change leaves the recombined fractions more susceptible to autoxidation.

In an effort to determine whether or not this stability lowering change was due to the acetone or Skellysolve F in which the phospholipids were dissolved, the phospholipids were removed by emulsification with water. As Table 5 shows, the phospholipids removed in this manner were even less effective in preventing oxygen uptake than were the acetone precipitated phosphotides. This strengthens the theory that the stability lowering change takes place as a result of the physical separation of the two fractions and not as a result of the solvent used.

Aside from what causes the decrease in the antioxygenic effectiveness of precipitated phospholipid, it is seen readily that its removal from the natural fat does not nearly account for the great drop in stability caused by converting the natural fat to monoesters. Since all of the evidence indicates that the resistance of the purified monoester to autoxidation equals or surpasses that of the corresponding triglyceride, there must be alterations other than the destruction of phospholipid which occur in the fat during the interesterification reaction to lower the stability of the esters.

SUMMARY AND CONCLUSIONS

1. The efficiency of various concentrations, ranging from 0.2 to 1.0 percent, of alpha and gamma tocopherol to prevent the autoxidation of synthetic chicken fat (monoesters) at 70 C. was investigated. For this temperature and substrate the optimum concentration for maximum stability of both forms of tocopherol was approximately 1 percent.

2. At their optimum concentrations for maximum stability, the gamma form of tocopherol was 5 times more effective than the alpha form in protecting the substrate from autoxidation.

3. Fats supplemented with gamma tocopherol did not have sharply defined induction periods; whereas those supplemented with alpha tocopherol did.

4. The methyl esters of chicken fat obtained by cold interesterification with methanol were 6 times less stable than the original fat. Experimental evidence indicated that the change in ester form was not responsible for the lower stability of the monoesters.

5. The stabilities of linoleic acid, trilinolein, and methyl linoleate increase in the order listed.

6. The phospholipid fraction of chicken skin tissue fat plays a definite role in protecting the fat from autoxidation.

7. Separating the phospholipid fraction from the natural fat greatly lowers the antioxygenic potency of the phospholipid when it is added back to the fat.

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